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The *RAD6* DNA Damage Tolerance Pathway Operates Uncoupled from the Replication Fork and Is Functional Beyond S Phase

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SUMMARY

Damaged DNA templates provide an obstacle to the replication fork and can cause genome instability. In eukaryotes, tolerance to damaged DNA is mediated largely by the *RAD6* pathway involving ubiquitylation of the DNA polymerase processivity factor PCNA. Whereas monoubiquitylation of PCNA mediates error-prone translesion synthesis (TLS), polyubiquitylation triggers an error-free pathway. Both branches of this pathway are believed to occur in S phase in order to ensure replication completion. However, we found that limiting TLS or the error-free pathway to the G2/M phase of the cell-cycle efficiently promote lesion tolerance. Thus, our findings indicate that both branches of the DNA damage tolerance pathway operate effectively after chromosomal replication, outside S phase. We therefore propose that the *RAD6* pathway acts on single-stranded gaps left behind newly restarted replication forks.

INTRODUCTION

DNA lesions that remain unrepaired before entering S phase pose a serious problem during replication. Besides a discontinuity of chromosomal replication, stalled replication forks are dangerous as they can collapse, causing chromosome breaks and genomic instability (Cox et al., 2000; Osborn et al., 2002). To cope with this problem, all organisms possess so-called DNA damage tolerance (DDT) pathways, which ensure cell survival in the presence of DNA polymerase-blocking lesions (Friedberg, 2005). Notably different from conventional DNA repair pathways, DDT does not result in repair of the primary DNA lesion but rather cures their symptoms that manifest during replication. DDT usually becomes activated as a result of a replication block-induced temporal uncoupling of DNA unwinding and synthesis (Chang and Cimprich, 2009). This leads to the formation of single-stranded DNA (ssDNA), a key trigger of DDT (Higgins et al., 1976; Little and Mount, 1982).

In bacteria, DDT appears to promote restart of stalled replication forks, which frequently involves repriming at the damaged template (Courcelle and Hanawalt, 2003). Interestingly, both pro- and eukaryotes utilize two distinct DDT modes: an error-prone mechanism, involving translesion polymerases that can bypass bulky DNA lesions by catalyzing DNA synthesis across the damaged template, and an error-free pathway that engages recombination proteins (Friedberg, 2005). As polymerases involved in translesion synthesis (TLS) can also incorporate an incorrect nucleotide across the damaged site, DDT is largely accountable for mutagenesis (Friedberg, 2005).

Distinctly different from the prokaryotic system, eukaryotic DDT requires the ubiquitin protein modification pathway, which does not exist in bacteria. Indeed, a large number of genes involved in eukaryotic DDT (called the *RAD6* pathway) encode enzymes of this protein modification system (Broomfield et al., 1998; Jentsch et al., 1987; Ulrich and Jentsch, 2000). The crucial substrate of this pathway is PCNA (Hoege et al., 2002), a homotrimeric, DNA-encircling protein, which functions as a DNA polymerase processivity factor and platform for replication-linked factors (Moldovan et al., 2007). Different types of ubiquitin modifications that become induced upon DNA damage dictate whether DDT proceeds via the error-prone or the error-free branch. Error-prone DDT is triggered by conjugation of a single ubiquitin moiety (monoubiquitylation) to PCNA at lysine-164 (K164), which involves the Rad6 ubiquitin-conjugating (E2) enzyme and Rad18, a RING-finger ubiquitin ligase (E3) that binds PCNA (Hoege et al., 2002; Stelter and Ulrich, 2003). Monoubiquitylated PCNA in turn promotes TLS possibly through direct recruitment of TLS polymerases that possess ubiquitin-binding motifs (Bienko et al., 2005; Kannouche et al., 2004; Lehmann et al., 2007; Watanabe et al., 2004). By contrast, error-free DDT requires modification of the same residue of PCNA by a polyubiquitin chain that is linked via K63 of ubiquitin (Hoege et al., 2002). Synthesis of this polyubiquitin chain requires in addition to Rad6 and Rad18 the heterodimeric E2 Ubc13-Mms2, and the RING-finger E3 ubiquitin ligase Rad5, which binds PCNA and Rad18 (Hoege et al., 2002; Ulrich and Jentsch, 2000). Once modified by this polyubiquitin chain, PCNA triggers by an unknown mechanism lesion bypass involving the undamaged template (template switching) and specific repair proteins (Branzei et al., 2008; Giot et al., 1997; Zhang and Lawrence, 2005). Furthermore, K164 of PCNA can alternatively be modified by the ubiquitin-related modifier SUMO (Hoege et al., 2002). In *S. cerevisiae*, this leads to the recruitment of Srs2, an antirecombinogenic helicase, which helps to keep at check an alternative error-free DDT mode that utilizes the Rad51 recombinase (Papouli et al., 2005; Pfander et al., 2005).

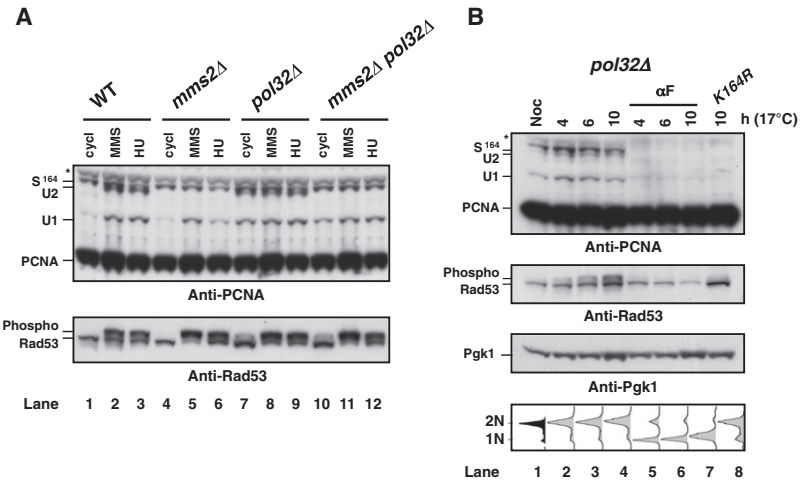

Figure 1. PCNA Ubiquitylation and Checkpoint Activation In *pol32*Δ Mutants

(A) Increased PCNA ubiquitylation and checkpoint activation in *pol32*Δ cells grown at permissive conditions. Cycling cultures (cycl; 30°C) were treated with 0.02% MMS (MMS) or 200 mM hydroxyurea (HU) for 2 hr, and whole-cell extracts were analyzed by western blot against PCNA and Rad53.

(B) Increased checkpoint activation (Rad53 phosphorylation) in *pol32*Δ at restrictive temperatures requires passage through S phase. Cells arrested in G2/M by nocodazole treatment were rapidly released and grown at 17°C in the absence (lanes 2–4) or presence of 10 μM α factor (lanes 5–7). Samples were withdrawn after 4, 6, or 10 hr and analyzed by western blot (Pgk1 used for loading control) and FACS (lower panel).

See also Figure S1.

Although DDT was initially coined "post-replicative DNA repair" (Howard-Flanders, 1968), the prevailing view today is that DDT acts directly at the replication fork in S phase (Andersen et al., 2008; Barbour and Xiao, 2003; Chang and Cimprich, 2009; Lee and Myung, 2008; Prakash et al., 2005; Ulrich, 2009). PCNA ubiquitylation is also believed to be physically coupled to stalled forks (Davies et al., 2008; Ulrich, 2009; Yang and Zou, 2009) and to promote fork progression (Bi et al., 2006; Leach and Michael, 2005). These and several other studies led to the broadly accepted model that TLS promotes "bypass replication" across the lesion at the replication fork, and that the error-free template-switching mode—either by sister chromatid junctions (SCJs) or fork regression leading to a DNA structure called "chicken foot"—acts near the replication fork, and promotes replication restart similar to bacterial DDT.

On the other hand, growing evidence has shown that a fraction of TLS can occur in the rear of the fork (Edmunds et al., 2008; Jansen et al., 2009a; Jansen et al., 2009b; Lopes et al., 2006; Waters and Walker, 2006). However, this issue still remains unsettled, as it was thus far not tested when and in which phase of the cell cycle the *RAD6* DDT pathway has to operate. This question is not only central from a mechanistic point of view, but also of general importance as DDT is crucial for cell survival upon DNA damage, genome stability, and tumor biology.

In this report, we test the present models directly by expressing key components of the error-prone and the error-free pathway specifically in the G2/M phase of the cell cycle. Surprisingly, they fully supported DDT virtually identical to wild-type (WT) cells. We also found that replication of damaged DNA continues and stalled replication restarts even in the absence of DDT. These findings strongly suggest that both branches of DDT in eukaryotes operate post chromosomal replication.

RESULTS

Replication Stress in *pol32*Δ Cells Activates the *RAD6* Pathway

Polymerase δ (Pol δ) plays an essential role in replication by catalyzing lagging-strand synthesis (Nick McElhinny et al., 2008). In budding yeast, it consists of two essential subunits, Pol3 and Pol31 (Hys2), and a small, nonessential subunit, Pol32 (Burgers and Gerik, 1998; Gerik et al., 1998). Although yeast cells lacking Pol32 (*pol32*Δ) proliferate well, they exhibit a delay at the G2/M phase of the cell cycle, which develops into a terminal G2/M arrest at low temperatures (Gerik et al., 1998; Huang et al., 1997; Huang et al., 1999). Notably, this phenotype is accompanied with phosphorylated checkpoint kinase Rad53 (Figure 1A, lane 7, and Figure S1A available online, lower panel) and the presence of nonsegregated chromosomes (Gerik et al., 1998; Huang et al., 2000), indicative of a DNA damage checkpoint-induced anaphase arrest (Sanchez et al., 1999). Importantly, similar to the temperature sensitivity of a subset of Pol δ mutants (Branzei et al., 2002; Giot et al., 1997; Vijeh Motlagh et al., 2006), the cold-sensitivity of *pol32*Δ cells can be efficiently suppressed by mutants in the *RAD6* pathway or by mutants expressing modification-deficient PCNA (*pol30*^{K164R}) (Figure S1B). We therefore conclude that this phenotype of *pol32*Δ cells largely depends on PCNA ubiquitylation.

PCNA ubiquitylation is barely detectable in unchallenged WT cells (Hoege et al., 2002) (Figure 1A and Figure S1A). However, we noticed that PCNA mono- and polyubiquitylation was strongly induced in *pol32*Δ mutants in S phase (Figures S1C and S1D). Interestingly, the level of ubiquitylated PCNA in *pol32*Δ cells was as high as in WT cells that have been exposed to methyl methanesulfonate (MMS) or hydroxyurea (HU) (Figure 1A). One possible explanation for this phenotype is that Pol32 may function as an enzymatic inhibitor of PCNA ubiquitylation. However, cells expressing a functionally compromised catalytic subunit of Pol δ (Pol3; *cdc2-2*) induced PCNA mono- and polyubiquitylation as well (Figure S1E). This indicates that faulty replication, rather than the absence of a specific polymerase subunit, is the cause that led to the induction of PCNA ubiquitylation in *pol32*Δ cells. Importantly, we also observed that *pol32*Δ cells at its restrictive temperature would not fully activate a DNA damage checkpoint response unless they were allowed to pass through S phase (Figure 1B). Thus, the primary defect of *pol32*Δ mutants originates during S phase, strongly suggesting that they experience replication stress. In fact, because the induced ubiquitylation of PCNA in *pol32*Δ cells could not be further enhanced by DNA-damaging agents

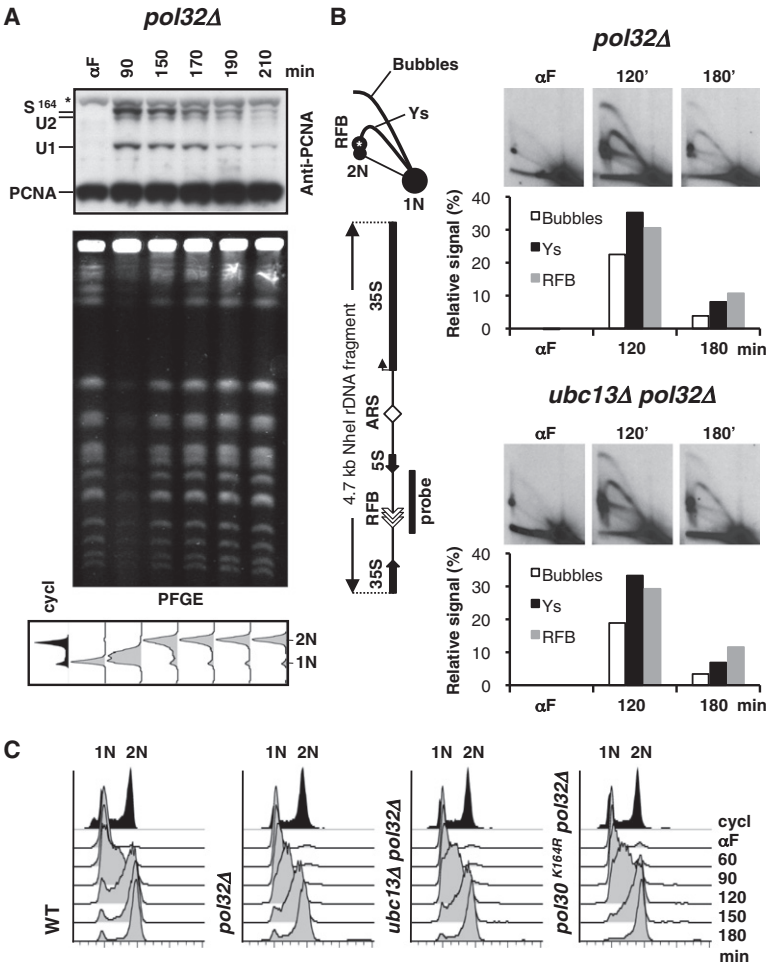

Figure 2. Ubiquitylated PCNA Persists beyond S phase and Is Not Required for S Phase Progression

Exponentially growing cells arrested by α factor and released in YPD at 17°C were collected for PFGE, FACS, and 2D gel analysis.

(A) *pol32*Δ cells show significant PCNA ubiquitylation still 190 min after α factor release (top panel) at a time when chromosomes have already been replicated (PFGE, middle panel) and passed S phase (FACS, bottom panel). Note that replicating chromosomes (at 90 min after release) cannot enter the PFGE gel (Lengronne et al., 2001).

(B) Replication of a late-replicating locus in *pol32*Δ cells is not affected by loss of PCNA polyubiquitylation. Genomic DNA was isolated from strains YGK1295 (*bar1*Δ *pol32*Δ) and YGK1297 (*bar1*Δ *ubc13*Δ *pol32*Δ), digested with NheI, and analyzed by 2D gel with a probe specific for the rDNA locus. Probe location and the definition of quantified signals are depicted in the schematic NheI rDNA fragment on the left. Asterisk indicates the replication fork barrier (RFB).

(C) S phase delay (assayed by FACS) of *pol32*Δ and *pol32*Δ cells additionally defective in PCNA ubiquitylation (*pol32*Δ *ubc13*Δ, *pol32*Δ *pol30*^{K164R}).

See also Figure S2.

(MMS or HU) (Figure 1A and Figure S1E), we conclude that most likely all active replication forks are affected in *pol32*Δ cells. This interpretation is in agreement with previous work showing that compromised Pol δ activity results in faulty replication with strong accumulation of ss gaps (Fukui et al., 2004; Johansson et al., 2004). Taking advantage of this phenotype, we used in this study *pol32*Δ mutants as a tool to analyze the function of the *RAD6* DDT pathway.

PCNA Ubiquitylation Accumulates behind Progressing Replication Forks

We noticed that although PCNA ubiquitylation in *pol32*Δ cells started accumulating in S phase, it remained high and a significant fraction persisted until the G2/M phase of the cell cycle (Figure 2A and Figure S1C). This considerable fraction of ubiquitylated PCNA in G2/M is apparently not associated with replication in S phase, as pulsed-field gel electrophoresis (PFGE) (Figure 2A) and fluorescence-activated cell sorting (FACS) analysis (Figure 2A and Figure S1C) revealed that the bulk of chromosomal replication was completed much earlier. In fact, at the time when replication was finished (as judged by cyclin Clb2 levels 340 min after α factor release at 14°C; Figures S1C and S1D), about 70% of ubiquitylated PCNA was still present and only slowly disappeared thereafter. We also followed replication progression in *pol32*Δ cells by visualizing replication intermediates by 2D gel electrophoresis. Notably, quantification of the intermediates showed that replication was largely (80%) completed before the onset of G2 even at a late-replicating locus (Brewer and Fangman, 1980) (Figure 2B, upper panels), at a time when PCNA ubiquitylation was essentially unaffected (Figure 2A). This lack of correspondence between the time of replication and the phase of the presence of ubiquitylated PCNA occurred not only in *pol32*Δ cells but also when PCNA ubiquitylation was triggered by sublethal doses of MMS or HU (data not shown). Interestingly, although SUMOylated PCNA started accumulating parallel to ubiquitylated PCNA in S phase, it vanished much faster (Figure 2A and Figure S1D), in agreement with the notion that PCNA SUMOylation may primarily act during S phase (Pfander et al., 2005). By contrast, the observed persistence of ubiquitylated PCNA beyond S phase suggests that the *RAD6* DDT pathway is possibly operational behind the replication fork.

Bulk DNA Replication in the Presence of DNA Damage Does Not Require Ubiquitylated PCNA

It has been proposed that, analogous to bacterial DDT, the eukaryotic DDT (*RAD6*) pathway also facilitates replication progression by promoting a restart of stalled forks (Barbour and Xiao, 2003). However, as our findings point to a role of PCNA ubiquitylation beyond S phase, we decided to revisit this issue.

We first addressed this question by using the *pol32*Δ mutation to induce faulty replication. Similar to other nonlethal Pol δ mutants, *pol32*Δ cells even at the permissive temperature progress through the cell cycle with an S phase delay (Figure 2C and Figure S2A), consistent with the interpretation that they endure replication stress. If the DDT pathway were needed for normal

replication of damaged templates, DDT mutants would be expected to accumulate replication intermediates. However, deletion of *UBC13* (required for PCNA polyubiquitylation) did not lead to an increase of replication intermediates (e.g., the "Y" structures measured at the ribosomal DNA [rDNA] locus in Figure 2B) in G2 in *pol32*Δ cells, as monitored by 2D gel electrophoresis of chromosomal DNA (Figure 2B). Moreover, also the duration of S phase remained apparently unaffected in the absence of PCNA ubiquitylation (Figure 2C). These finding clearly indicate that a functional DDT pathway is not needed for normal S phase progression in the presence of (e.g., *pol32*Δ-induced) replication stress.

We also induced replication stress by treating cells with MMS or HU to monitor genome-wide responses. Notably, cells deficient in DDT progressed through S phase with WT kinetics (Figure S2A), even when they replicate in the presence of MMS (Figure S2B). To get additional support for the model that the DDT pathway is not needed for the completion of replication of damaged templates we took advantage of an assay that monitors replication restart. In this setup, cells were first arrested in G1 with α factor, released from the arrest into HU-containing medium to stall replication, and then incubated in an HU-free medium (Figure S2C). In fact, we found that mutants that lack DDT activity (*mms2*Δ *rev3*Δ) were proficient in stalled fork restart after transient HU treatment, as judged by PFGE and FACS analysis (Figure S2C; left and central panel).

Lastly, we also monitored stalled replication by following the level of the phosphorylated form of Mrc1 (yeast claspin), which is thought to be an indicator for stalled replication forks (Alcasabas et al., 2001; Osborn and Elledge, 2003). We confirmed that HU-treated cells carrying a deletion of the gene for Mrc1 progress faster through S phase than the WT, consistent with the role of Mrc1 in the checkpoint response (Figure S2C, right panel). If the DDT pathway was needed for reactivating stalled replication forks, an accumulation of phosphorylated Mrc1 would be expected in DDT mutants. However, we found that the induction and decay of phospho-Mrc1 was unaffected by the absence of an active DDT pathway (Figure S2D). Thus we conclude that contrary to previous models, PCNA ubiquitylation and DDT are not required for S phase progression and replication restart in the presence of damaged templates.

TLS Efficiently Operates during G2/M

We next asked whether PCNA ubiquitylation plays a crucial role after replication. Interestingly, although mutants defective in PCNA ubiquitylation showed no delay in S phase after MMS treatment, they exhibited a strong increase in the activation of the DNA damage checkpoint (Figure 1A and Figure S2B). This indicates that the DDT pathway prevents the accumulation of damaged DNA but is apparently not crucial for S phase progression. We therefore tested whether the *RAD6* DDT pathway operates in G2/M.

TLS in *S. cerevisiae* is conducted in a collaborative manner by the TLS polymerases Rev1, Pol ζ (comprised of the subunits Rev3 and Rev7) and Pol η /Rad30, and TLS induced by DNA damage additionally requires PCNA monoubiquitylation (Hoege et al., 2002; Kunz et al., 2000; Lehmann et al., 2007; Stelter and Ulrich, 2003). To address whether TLS can occur after S phase has been completed, we limited the expression of TLS polymerases to the G2/M phase of the cell cycle by taking advantage of the regulatory elements of the mitotic cyclin Clb2. This protein is expressed exclusively in G2/M and rapidly degraded by the ubiquitin-proteasome system (via D- and KEN-box degrons) at the end of mitosis and in G1 (Wäsch and Cross, 2002). We inserted the *CLB2* promoter and sequences encoding the N-terminal 180 amino acids (bearing the degrons, a L26A replacement to prevent nuclear export (Hood et al., 2001), plus a Clb2-derived antibody epitope) of Clb2 (together termed G2 tag) in front of the open reading frame of *REV3* and *RAD30* at their respective genomic loci. The resulting cells thus only express the designed fusions, and we termed the alleles *G2-REV3* and *G2-RAD30* (and the proteins G2-Rev3 and G2-Rad30) in the following (Figure 3A). Analysis of synchronized cultures showed that expression of *G2-REV3* and *G2-RAD30* was indeed restricted to G2/M identical to that of Cbl2 (Figure 3B and Figure S3A) even after short MMS treatment (Figure S3B). In addition, after release from nocodazole-induced G2/M arrest, degradation of the protein fusions was highly similar and parallel to that of Clb2 itself (Figure S3C).

G2-REV3 rescued the phenotypes of the corresponding *rev3*Δ null mutant toward ultraviolet (UV) light, MMS, and 4-nitroquinoline 1-oxide (4NQO) (Figure 3C). Similarly, expression of *G2-RAD30* also restored resistance of *rad30* knockout cells toward UV light and MMS to WT levels (note that *rad30*Δ is not sensitive to 4NQO; Figure 3C), and cells that coexpress *G2-REV3* and *G2-RAD30* also show WT sensitivities toward MMS (Figure S3D). As has been noted before (Broomfield et al., 1998; Torres-Ramos et al., 2002), mutants deficient in *REV3* and *RAD30* show synergistic phenotypes with *mms2*Δ (Figure S3D), and these phenotypes were indeed also suppressed by *G2-REV3* and *G2-RAD30* (or even in combination) (Figure S3D).

Since the error-prone TLS polymerase Rev3 is involved both in spontaneous and induced mutagenesis (Hastings et al., 1976; Lawrence and Christensen, 1976; Morrison et al., 1989), we also examined these functions of *G2-REV3* by assaying mutations at the *CAN1* locus. Indeed, *G2-REV3* was proficient in spontaneous mutagenesis in *mms2*Δ cells (Figure 3D). It also supported induced mutagenesis as the construct could fully complement the defect in UV light-induced mutagenesis of *rev3*Δ (Figure 3E). Notably, this complementation also occurred in the absence of the error-free TLS polymerase Rad30 (Figure 3E).

We further tested *G2-RAD30*-expressing cells for mutator phenotypes. Cells deficient in the error-free TLS polymerase Rad30 (*rad30*Δ) show a mutator phenotype when exposed to UV light because the error-prone TLS Pol ζ takes over (McDonald et al., 1997). Notably, this phenotype is also suppressed by *G2-RAD30* (Figure 3E), indicating that *G2-RAD30* behaves like WT *RAD30* also in this function.

Lastly, we also tested the proficiency of G2-restricted TLS by using components of the PCNA ubiquitylation machinery. Indeed, *G2-RAD18* was able to restore UV survival and induced mutagenesis in cells deficient in PCNA SUMOylation and polyubiquitylation (Figure S3E). Importantly, mutagenesis in *G2-RAD18*-expressing cells strictly required K164 of PCNA

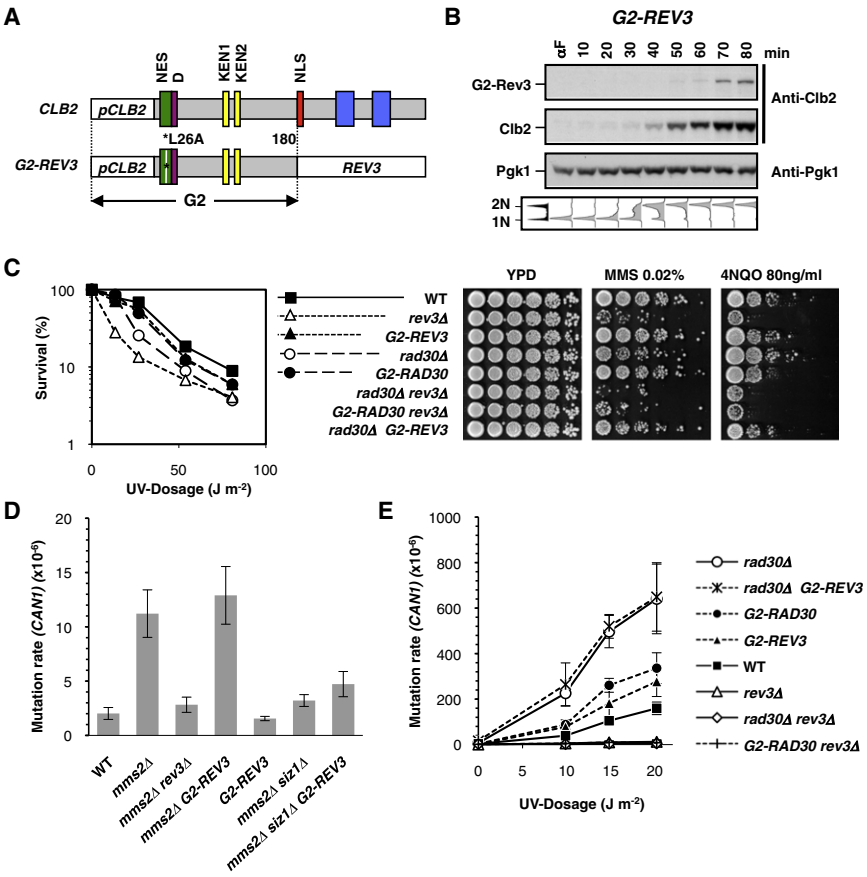

Figure 3. TLS Efficiently Operates during G2/M

(A) Elements of the Clb2 protein and gene (upper panel) and the *G2-REV3* chimera. D (violet) and KEN boxes (yellow), nuclear export (NES, green) and nuclear localization (NLS, red) sequences, and two CYCLIN domains (blue) are indicated. The *REV3* gene (and other DDT genes used in this study) was fused to the "G2 tag," a DNA sequence that carries the *CLB2* promoter (*pCLB2*; which is activated at G2/M) and sequences encoding the N-terminal 180 amino acids of Clb2 harboring the D- and KEN box degrons (the NES is mutated; L26A, indicated by an asterisk). The G2 tag encoded peptide also reacts with the Clb2 antibody.

(B) G2-Rev3 is expressed specifically during G2/M. Western blot analysis (Clb2 antibody) of the G2-Rev3 TLS polymerase fusion upon release from α factor arrest at 23°C. Pgk1 levels were used as loading control. Lower panel shows FACS profiles for the same samples.

(C) G2-TLS polymerase fusions complement the sensitivities of their corresponding deletion mutants to UV light (left panel), MMS, and 4NQO (right panel). Spotting of 1:5 serial dilutions on YPD plates supplemented with MMS or 4NQO.

(D and E) G2-TLS polymerase fusions are proficient in catalyzing TLS. Rates of spontaneous (D) and UV-induced (E) mutagenesis are shown for the *CAN1* locus. Values and associated error bars represent averages and their standard deviations from 3-7 independent experiments.

See also Figure S3.

(Figure S3E), verifying that this function in G2/M is mediated specifically by monoubiquitylated PCNA. Thus, our findings demonstrate that both Rev3 and Rad30 as well as monoubiquitylated PCNA operate efficiently on damaged templates even when restricted to G2/M. By extension, we conclude that TLS triggered by PCNA monoubiquitylation, and also spontaneous PCNA ubiquitylation-independent TLS (Stelter and Ulrich, 2003), does not normally mediate bypass replication at stalled replication forks, but rather operates behind replication forks, even outside of S phase.

A Genetic Screen for Factors Involved in Error-free DDT

As the cold sensitivity of *pol32*Δ cells depends largely on PCNA polyubiquitylation (Figure S1B), we took advantage of this phenotype for a screen for genes that function in error-free DDT. Specifically, we asked whether we could identify mutants that can exacerbate the phenotype of *pol32*Δ in a manner dependent on PCNA polyubiquitylation. By using a robot-based platform, we screened for deletion mutants that are lethal at 23°C in combination with *pol32*Δ. Because we conducted in parallel the same screen with cells additionally deficient in *MMS2*, we were able to identify synthetic lethal mutants of *pol32*Δ that can be rescued by deletion of additionally *MMS2* (Figure S4A). We conjectured that such mutants are defective in activities that are triggered by PCNA polyubiquitylation. Among the few identified *pol32*Δ synthetic lethal mutants that fulfilled this criterion was *SGS1* (Figures S4B and S4C), which encodes a RecQ-type helicase related to the WRN and BLM enzymes mutated in the human disorders Werner and Bloom syndrome, respectively (Nakayama, 2002). As these enzymes exercise important roles during DNA replication (Bachrati and Hickson, 2008; Sidorova et al., 2008), stalled fork stabilization and restart (Cobb et al., 2003; Stewart et al., 1997), and recombination (Karow et al., 2000; Liberi et al., 2005; Mimitou and Symington, 2009; Wu and Hickson, 2003) we decided to investigate the basis behind the reduced viability of the *pol32*Δ *sgs1*Δ strain. Notably, the ability of *pol32*Δ cells to ubiquitylate PCNA and activate the DNA damage checkpoint (as monitored by Rad53 phosphorylation and FACS analysis) were not affected by the absence of *SGS1*, as assayed in an *SGS1*-expression shut-off experiment (glucose-repressible *GAL*-promoter; Figure S4D). Moreover, *SGS1*-depleted *pol32*Δ cells, just like *pol32*Δ single mutants, generated no significant levels of double-strand breaks at low (restrictive) temperatures (Figure S4E). Altogether, these findings indicate that stalled fork stabilization may not be the underlying activity behind the lethality of *pol32*Δ *sgs1*Δ cells.

In agreement with the view that the essential function of Sgs1 in the absence of Pol32 is not linked to the replication fork, we obtained evidence that the basis for the low viability of *pol32*Δ *sgs1*Δ cells and the cold sensitivity of *pol32*Δ cells are alike. First, *pol32*Δ cells depleted for Sgs1 also showed increased activation of the DNA damage checkpoint, as judged by the

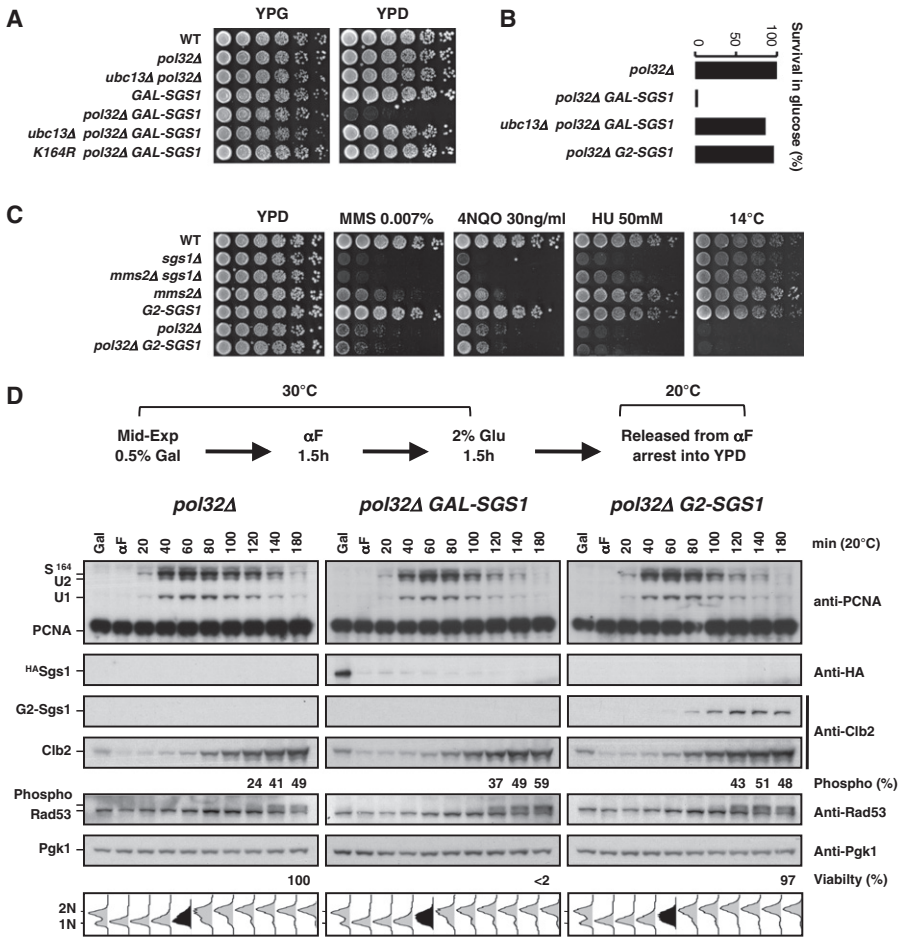

Figure 4. Sgs1 Promotes Error-free DDT Effectively in G2/M

(A) Synthetic lethality of *pol32*Δ *sgs1*Δ depends on PCNA polyubiquitylation. *SGS1* (tagged with HA epitope) driven by the *GAL* promoter (*GAL-SGS1*) was integrated at its genomic locus and turned off by plating on glucose-containing media (right panel). Serial 1:5 dilutions were spotted on plates and incubated at 23°C for 2.5 days.

(B) Restriction of Sgs1 to G2 (*G2-SGS1*), but not Sgs1 depletion (*GAL-SGS1*), supports viability in the absence of *POL32*. Cells from cultures grown to logarithmic phase (YP medium, 0.5% galactose 1.5% raffinose) were plated on galactose- (YP, 2% galactose) or glucose- (YP, 2% glucose) containing plates, incubated at 30°C for 3 days, and counted.

(C) Sgs1 promotes PCNA polyubiquitylation-dependent DDT effectively at G2/M.

(D) Removing Sgs1 from S phase does not affect PCNA modification or viability in the presence of replication stress. Protein profiles of *pol32*Δ cells, *pol32*Δ cells depleted for Sgs1 (*GAL-SGS1*), and *pol32*Δ cells expressing Sgs1 in G2/M (*G2-SGS1*). Cells were grown to early exponential phase in the presence of 0.5% galactose and 1.5% raffinose, arrested in G1 with α factor (1.5 hr, 30°C) (Gal), and subsequently supplemented with 2% glucose (to shut off *GAL-SGS1* expression; see scheme). After additional 1.5 hr incubation, cultures were washed and grown in YPD (2% glucose) at 20°C. Samples withdrawn at different time-points were probed by western analysis for the indicated proteins. The lower panel shows FACS profiles for the same samples and (above the panel) cell survival after plating on 2% glucose YP-plates.

See also Figure S4.

phosphorylation of Rad53 (Figure S4D). Second, we found that *pol32*Δ *sgs1*Δ was rescued to almost WT growth not only by *mms2*Δ, but also by other mutants in the *RAD6* pathway (Figures 4A and 4B), suggesting that PCNA polyubiquitylation is indeed responsible for the reduced growth of the double mutant. Furthermore, the MMS sensitivities of the *sgs1*Δ single mutant and the *sgs1*Δ *mms2*Δ double mutant were identical (Figure 4C), indicating that *SGS1* and *MMS2* function in the same pathway. Indeed, a recent study has shown that the role of Sgs1 in error-free DDT is the resolution of X-shaped SCJs that form upon replication of damaged templates (Branzei et al., 2008).

When we assayed for the effect of drugs that particularly lead to ss gap accumulation like HU and 4NQO, we observed a partial rescue of the sensitivities of *sgs1*Δ cells by *mms2* mutants deficient in PCNA polyubiquitylation (Figure 4C). This rescue depends on homologous recombination (data not shown). These results suggest that ss gaps (spontaneously arising in *pol32*Δ or induced by exogenous DNA damage) through the action of polyubiquitylated PCNA induce toxic or persistent recombination

intermediates that are normally dissolved (detoxified) by the Sgs1/Top3/Rmi1 complex.

Restricting Sgs1 to G2/M Fully Supports Error-free DDT

After having identified Sgs1 as a crucial mediator of error-free DDT, we next asked whether this pathway is operational in G2/M as well. Indeed, when we constructed a G2 fusion with *SGS1* (*G2-SGS1*) and expressed it as the only source of Sgs1 in *pol32*Δ cells, we observed that they formed colonies like *pol32*Δ cells expressing WT Sgs1, whereas *sgs1*Δ *pol32*Δ double mutants are inviable (Figure 4B). Moreover, *G2-SGS1*-expressing cells exhibited no hypersensitivity toward DNA-damaging agents compared to WT even in the absence of *POL32* (Figure 4C). We next compared cell-cycle progression and checkpoint activation of *pol32*Δ cells grown at (semipermissive) 20°C with *pol32*Δ *G2-SGS1* cells and with *pol32*Δ cells depleted for Sgs1 (*GAL-SGS1*). In this experiment, we released cells from an α factor arrest and monitored synchronous cell-cycle progression in the presence of glucose (Figure 4D, top). We found that DNA damage checkpoint activation (monitored by Rad53 phosphorylation) was evidently higher in Sgs1-depleted *pol32*Δ cells than in *pol32*Δ and *pol32*Δ *G2-SGS1* cells (Figure 4D; compare the 180 min lanes), which paralleled their inability to form colonies on plates (Figure 4D). This suggests that Sgs1 restricted to the G2/M phase of the cell cycle indeed represses *pol32*Δ-induced DNA damage checkpoint activation similar to WT Sgs1.

We noticed that Rad53 phosphorylation in *pol32*Δ *GAL-SGS1* and *pol32*Δ *G2-SGS1* cells occurred about 20 min earlier than in *pol32*Δ cells (Figure 4D). This was due to a faster progression through the cell cycle of the first two cultures than in *pol32*Δ cells, as determined by FACS analysis (Figure 4D). Notably, faster S phase progression was shown before for *sgs1*Δ cells grown in the presence of the HU (Frei and Gasser, 2000), which led to the suggestion that Sgs1 plays a role in promoting the S phase checkpoint. Thus we assume that the absence of Sgs1, or its restriction to G2/M, also weakens S phase checkpoint activation in *pol32*Δ, thereby causing the observed faster S phase progression. Notably, this defect in S phase checkpoint activation resulted in no increased MMS or HU sensitivities (Figure 4C). In conclusion, while G2/M-restricted Sgs1 fully supports its vital role in the error-free DDT pathway, it does not seem to support its other, DDT-unrelated function in S phase. Importantly, this finding also demonstrates the nonleakiness of the G2 tag.

Rad5 Fully Functions in DDT When Restricted to G2/M

As the above findings demonstrate that the activities of both *RAD6* DDT branches are operational after S phase, we next asked whether the functions of the ubiquitylation enzymes that initiate DDT could be restricted to G2/M as well. Indeed, we found that *G2-RAD18*, *G2-UBC13*, and *G2-RAD5* complemented the DNA damage sensitivities to MMS and 4NQO of the corresponding knockout mutants almost completely (Figure 5A).

We focused on Rad5 because it not only catalyzes PCNA polyubiquitylation (Hoege et al., 2002), but also controls a large part of TLS events (Lemontt, 1971) and possesses helicase activity (Johnson et al., 1994). Notably, all three activities are involved in DDT (Gangavarapu et al., 2006), and it has been proposed that the helicase activity supports DDT through mediating fork regression (Blastyák et al., 2007). However, this suggested role of Rad5 seems unlikely given the fact that *rad5*Δ cells were fully capable of replicating through MMS-damaged DNA without delay (Figure 5B). On the other hand, *rad5*Δ mutants strongly activated the DNA damage checkpoint (Figures 5B and 5C) and exhibited a delay in reentering the cell cycle (as visualized by an increase in PCNA SUMOylation; Figure 5B, upper panel) and reduced viability (Figure 5D). Notably, PFGE-analyzed chromosomes from MMS-treated *rad5*Δ cells (but not WT cells) remained heat labile (produced DSBs) in vitro long after their replication had been completed (Figure 5E). It has been noticed before that fragile chromosomes arise after MMS treatment (Lundin et al., 2005), and hence we deduce that the observed *rad5*Δ phenotype is the result of accumulated ss gaps generated by replication through MMS-damaged DNA templates.

When we analyzed a synchronously growing culture of *G2-RAD5* cells, we found that the enzyme promoted PCNA polyubiquitylation upon MMS-induced DNA damage specifically in the G2/M phase (Figure 5B and Figure S5A), matching its restricted expression in this phase of the cell cycle. This result is in disagreement with a model in which Rad5 becomes activated only during replication upon stalled replication forks. The construct also supported the function of Rad5 in DDT (Figure 5A and 5D) and prevented the MMS-induced generation of heat-labile chromosomes (Figure 5E), indicating that ss gaps are repaired if Rad5 is restricted to G2/M. *G2-RAD5* also effectively complemented MMS-induced checkpoint activation (Rad53 phosphorylation) later during G2/M phase (when the fusion is expressed; Figures 5B and 5C; 60 min and later), thus accelerating entry into the following cell cycle (Figure 5B, upper panel). However, *G2-RAD5* was unable to prevent checkpoint activation earlier (Figures 5B and 5C; 45–60 min), demonstrating that G2-Rad5 is not present during S phase (demonstrating the ''nonleakiness'' of the G2 tag). Importantly, the cold sensitivity of *pol32*Δ mutants (which depends on the *RAD6* pathway including Rad5; Figure S5B), the MMS sensitivity of *ubc13*Δ cells (Figure S5C), and spontaneous (Figure S5D) and UV-induced (Figure S5E) mutagenesis remained unaltered when G2-Rad5 was expressed as the only source of Rad5. From these findings, we conclude that the construct specifically promotes bona-fide error-free DDT via PCNA polyubiquitylation. Thus, MMS- and *pol32*Δ-caused DNA defects, although they originate in S phase, can be sensed and processed by Rad5-dependent DDT after S phase. In conclusion, our data indicate that PCNA polyubiquitylation and error-free and error-prone DDT are all fully functional postreplicatively outside of S phase and are therefore not required for S phase completion.

DISCUSSION

The highly conserved *RAD6* pathway plays two crucial roles in life. On the one hand, it provides tolerance to replication stress caused e.g., by DNA lesions that may stall or slow down DNA replication. On the other hand, because one of its modes involves error-prone translesion polymerases, it is accountable for de novo mutations and hence relevant for carcinogenesis

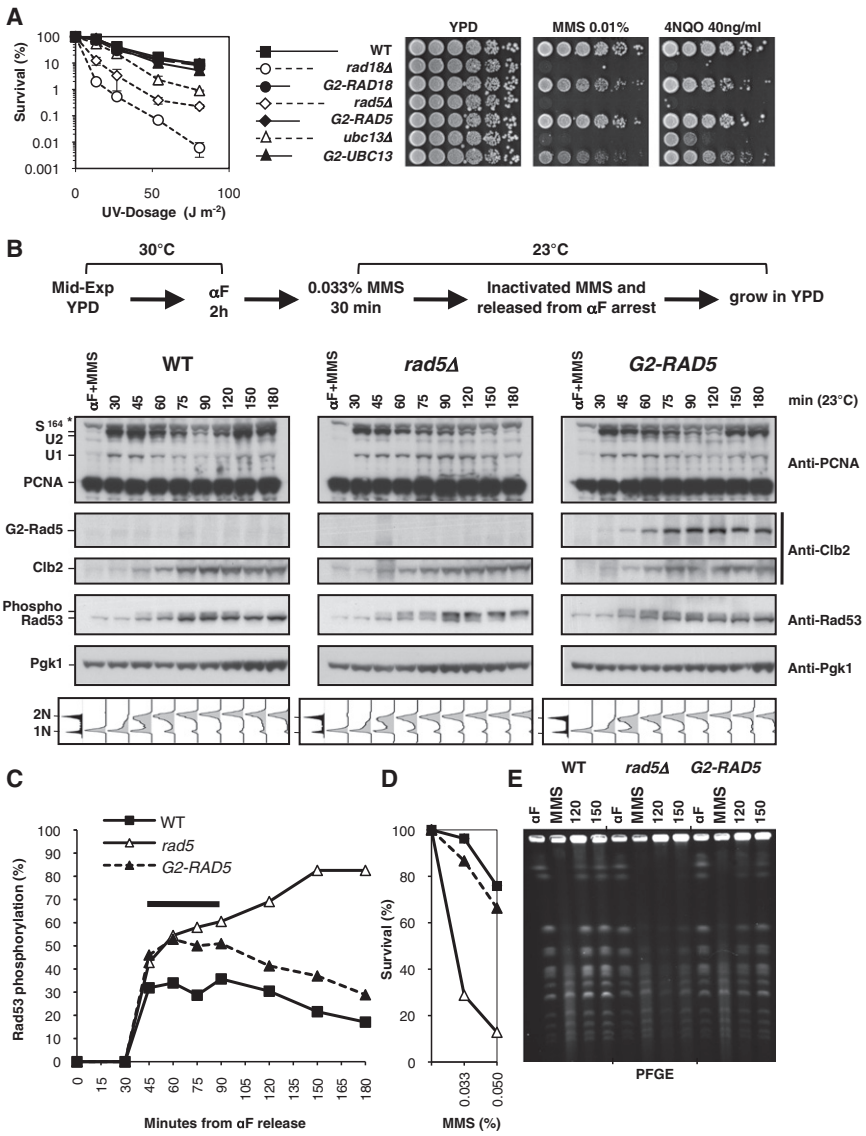

Figure 5. PCNA Polyubiquitylation in G2/M Supports DDT

(A) G2 fusions of Rad18, Rad5, and Ubc13 complement the sensitivity of their respective deletion mutants to UV light (left panel), and DNA-damaging agents (1:5 serial dilutions on YPD containing MMS, HU, or 4NQO; right panel). Error bars represent standard deviations from two independent experiments.

(B–E) WT, *rad5*Δ, and *G2-RAD5*-expressing cells were arrested in G1 (α factor, 30°C), transferred to 23°C, and treated with MMS (0.033%; additionally 0.05% for Figure 5D) for 30 min (see scheme). Subsequently, MMS was inactivated by Na-thiosulfate (5%) and cells were synchronously released from G1 arrest by transfer into YPD medium. Samples before (α factor) and after release were withdrawn at the indicated timepoints and analyzed by western blots, FACS, and PFGE.

(B) G2-Rad5 (detected with Clb2 antibody; *G2-RAD5*) accumulates parallel to Clb2 in G2/M (time points 60–180 min) and after S phase completion (FACS; lower panel) and supports PCNA polyubiquitylation during G2/M (time points 60–90 min; see enlargement of the anti-PCNA blot in Figure S5A).

(B and C) Phosphorylated Rad53 increasingly accumulates in *rad5*Δ cells in G2/M (anti-Rad53 blot; 45–180 min). Although *G2-RAD5*-expressing cells reach similar phospho-Rad53 levels during S-G2 (45–60 min), they drop to almost WT levels yet delayed by about 30 min (bar).

(C) Quantification of the phospho-Rad53 levels detected by western blots.

(D) Survival of cells from the same experiment before or after MMS addition (plated on YPD and colonies were counted after 2 days at 30°).

(E) PFGE analysis of the same samples before (α factor) and after MMS treatment (MMS), and 120 and 150 min after G1 release. Chromosomes from MMS-treated cultures are labile upon heat treatment in vitro and appear fragmented after PFGE (Lundin et al., 2005).

See also Figure S5.

(Friedberg, 2005), but also somatic hypermutation (Arakawa et al., 2006), genetic variation, and evolution.

DDT Is Operative after S Phase

To test the broadly accepted model that the *RAD6* pathway needs to function directly during S phase, we developed the G2 tag to restrict the expression of crucial DDT components to the G2/M phase of the cell cycle. Importantly, characteristic activities that need to function during S phase were not supported if the proteins were expressed by this system, demonstrating that the G2 tag is not "leaky" but accurate for expression in the G2/M phase.

Despite this specificity, tolerance to DNA damage in G2/M may theoretically still take place on forks that possibly have escaped S phase and persisted until G2/M (Weinert, 2007). However, the number of residual forks already in late S is apparently very low in unchallenged cells (Raghuraman et al., 2001) (D. Collingwood, M. Raghuraman, and B. Brewer, personal communication). The same holds true for a late-replicating locus under conditions of replication stress (*pol32*Δ; Figure 2B), which does not alter the order in the genome-wide replication program (Alvino et al., 2007). Thus, it seems unlikely that a diminutive fraction of forks in G2 can account for all DDT activities (DNA damage tolerance and mutagenesis) triggered by several hundred of stalled forks throughout S phase (Feng et al., 2006).

By using the G2 tag, we found that the otherwise metabolically stable TLS polymerases Pol η/Rad30 and Rev3 were fully capable of supporting survival in the presence of DNA-damaging agents and mutagenesis when restricted to the G2/M phase of the cell cycle. This indeed suggests that TLS acts predominantly on DNA lesions that stay behind the moving fork. This conclusion is in line with previous findings in yeast showing that TLS polymerases counteract the formation of gaps behind replication forks without affecting fork progression (Lopes et al., 2006)

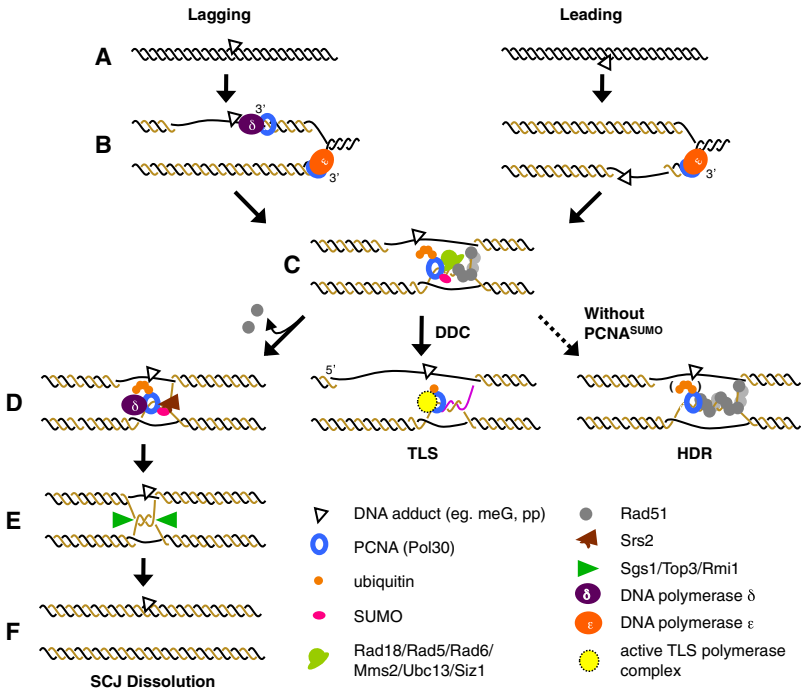

Figure 6. Hypothetical Model for Tolerance to Replication-Blocking DNA Lesions

Bulky DNA adducts (e.g., methylated bases and photoproducts) on the template strand (black) block DNA synthesis by the replicative polymerases (A). DDT is activated in an identical manner by lesions blocking the lagging-strand (B, left; Pol δ) or the leading-strand polymerases (B, right; Pol ε). Leading-strand synthesis (yellow) stalling promotes repriming downstream of the lesion, thereby leaving a ssDNA stretch (ss gap) that contains the lesion behind. Similarly, ss gaps are formed by lagging-strand stalling (incomplete Okazaki fragments). The ss gaps participate in D loop formation (C; boxed figure), which are resolved by three different mechanisms. Modification of PCNA (blue ring) by SUMO (crimson) triggers recruitment of the helicase Srs2 (brown triangle), which removes the recombinase Rad51 (gray) from chromatin. The first mechanism (left row; D–F) involves polyubiquitylated (orange) PCNA and sister chromatid junctions (SCJs), which are dissolved by the Sgs1-Top3-Rmi1 complex (green triangles). The second mechanism (middle row) is activated by the DNA damage checkpoint (DDC) and involves PCNA monoubiquitylation, which triggers the recruitment of specific TLS polymerases for induced TLS. The third mechanism (right row) is activated in the absence of PCNA SUMOylation (without $PCNA^{SUMO}$). This pathway involves Rad51-dependent recombination and the resolution of double Holliday junctions (dHJ) involving the Sgs1-Top3-Rmi1 complex (green) or specific nucleases.

and that Rev1 is strongly enriched during G2/M (Waters and Walker, 2006). Thus, it seems that at least yeast TLS polymerases operate usually outside bulk replication and that switching from replicative to TLS polymerases may not normally function at stalled replication forks. This situation appears to be different in higher eukaryotes in which Rev1 and Polζ TLS polymerases have functions at the replication fork (Edmunds et al., 2008; Jansen et al., 2009a, 2009b).

Our genetic screen and previous work (Branzei et al., 2008) identified the RecQ-type helicase Sgs1 as an essential downstream component of the error-free branch of DDT. Sgs1 (homologous to the human WRN and BLM proteins) functions to dissolve topological DNA structures such as supercoils and catenanes. Indeed, we found that also PCNA polyubiquitylation and Sgs1 activity support error-free DDT if restricted to G2. This finding directly challenges the view that Rad5 is needed for fork regression during DDT. Moreover, it gives strong support to the model that error-free DDT operates instead via template switching involving SCJs, apparently across lesions that stay behind the moving replication fork (Branzei et al., 2008; Liberi et al., 2005; Lopes et al., 2006).

A Postreplication Fork Model For DDT

It has been argued previously that the *RAD6* pathway is exclusively linked to leading-strand synthesis (Gangavarapu et al., 2007). However, we discovered that cells lacking Pol32, a subunit of Pol δ required for lagging-strand synthesis, also generate lesions that induce PCNA mono- and polyubiquitylation and are processed by the *RAD6* DDT pathway. Thus, we rather propose that the *RAD6* pathway becomes activated both by stalled leading- or lagging-strand synthesis. We and others (Branzei et al., 2006, 2008; Liberi et al., 2005; Lopes et al., 2006) found that bulk replication continues without delay in the absence of either error-prone or error-free components. Because the stresses applied are expected to impair most active forks (cells exposed to 0.033% MMS for 30 min contain at least one lesion per 20 kb according to Ma et al. [2009]); for *pol32*Δ cells, see the Results), and since *RAD6* tolerates DNA damage in a genome-wide manner (Lawrence et al., 1974), the *RAD6* pathway does not seem to be needed for S phase completion and for restarting stalled replication. It also has been suggested that PCNA ubiquitylation may take place directly at stalled forks (Davies et al., 2008; Ulrich, 2009). However, by using the G2 tag, we found that PCNA can be efficiently ubiquitylated even at a time when the bulk of replications forks have encountered DNA lesions.

Thus, the key feature of our model (Figure 6) is that the *RAD6* pathway operates in the rear of continuing replication, but not directly at the replication fork. In this model, bulky lesions that block leading- (Pol ε) or lagging- (Pol δ) strand DNA synthesis activate DDT. Leading-strand synthesis stalling promotes repriming beyond the lesion, thereby leaving a ss gap containing the lesion behind (Lehmann and Fuchs, 2006). Conversely, lagging-strand synthesis stalling causes gaps due to incomplete Okazaki fragment synthesis. These discontinuities trigger checkpoint activation and in parallel ubiquitylation of PCNA molecules located (loaded) at the lesion. In our model, the formation of a D loop, which engages the undamaged sister duplex and the free 3′ end of a ss gap, is crucial for all branches of DDT. In the absence of PCNA SUMOylation, this structure is the substrate

for Rad51-mediated sister chromatid recombination (Papouli et al., 2005; Pfander et al., 2005). Polyubiquitylated PCNA may promote gap filling and the subsequent formation of SCJs that will be finally dissolved by the Sgs1/Top3/Rmi1 complex. By contrast, monoubiquitylated PCNA promotes TLS through recruitment of TLS polymerases that bypass the lesion post-replicatively in an error-free or error-prone manner.

It has been suggested that polyubiquitylation of PCNA may follow its monoubiquitylation (Andersen et al., 2008). However, we propose that error-free DDT usually precedes mutagenic events. A crucial argument comes from the finding that by restricting the PCNA polyubiquitylation enzyme Rad5 to G2/M, the ability of newly polyubiquitylated PCNA to hold back MMS-induced checkpoint activation was delayed (Figures 5B and 5C and Figure S5A). This suggests that, although not necessarily coupled to replication, error-free DDT may usually commence during S phase and continues if needed during G2/M. This is clearly different to TLS, as we found no competition between error-prone and error-free DDT of spontaneous lesions by restricting Rad5 to G2/M (Figure S5D). This implies that TLS-mediated DDT requires in addition to PCNA polyubiquitylation a second signal. In fact, TLS (but not error-free DDT) seems to be stimulated by the Mec1/ATR-dependent checkpoint response (Pagès et al., 2009; Paulovich et al., 1998; Sabbioneda et al., 2007; Sabbioneda et al., 2005). As TLS operates effectively outside S phase, the checkpoint pathway in question is not the conventional S phase checkpoint but rather the DNA damage checkpoint that becomes activated by the DNA gaps that accumulate behind the replication fork. This model also suggests that the choice between TLS and error-free DDT may be ultimately dictated by the type of lesions, i.e., whether the gaps induce the DNA damage checkpoint or not.

Conclusions

The primary raison d'être for a postreplicative DDT mode is most likely the ability to uncouple chromosomal replication from means that take care of DNA synthesis-blocking lesions. Restarting replication forks beyond the lesion appears to be a simple and fast way to continue replication. This might be especially relevant for eukaryotes as eukaryotic replication involves numerous origins. If DDT had to operate exclusively at the stalled fork, a few lesions would already be expected to delay genomic replication, or cause genomic instability. Moreover, this uncoupling may also be beneficial for DDT itself as it makes additional time for the DDT mechanisms to cope with the lesions. Furthermore, replication fork uncoupled DDT may facilitate desired choices between error-free DDT and mutagenesis, perhaps guided by the sizes of the ss gaps located opposite of the D loops. By evolving replication fork-uncoupled DDT pathways, eukaryotes may have acquired more effective means to control mutagenesis for desired hypermutation (e.g., at the IgG locus), genetic adaptation, and speciation.

EXPERIMENTAL PROCEDURES

S. cerevisiae Strains

Yeast strains used in this study are listed in Table S1.

Estimation of Mutation Rates

Spontaneous and UV-induced forward mutagenesis at the *CAN1* locus were assessed by measuring the canavanine resistant fraction of parallel saturated populations. UV irradiation was performed in glass petri dishes using 6×10^8 cells. Averages were obtained from two to seven independent experiments.

FACS Analysis and Pulse-Field Gel Electrophoresis

Cells were stained for DNA content with SYTOX or propidium iodide (see the Extended Experimental Procedures). Intact chromosomes were extracted from cells embedded in agarose plugs, run on agarose gels using a pulse field apparatus, and visualized by ethidium bromide staining.

2D Gel Analysis of Replication Intermediates

Synchronously growing cells ($1.5–2 \times 10^9$) were treated with 0.1% sodium azide and vortexed with glass beads, and genomic DNA was purified on cesium chloride gradients and digested with NheI. First-dimension gels (0.4% agarose) were run for 22 hr, and second-dimension gels (1% agarose) were run for 4.5 hr at 4° in the presence of ethidium bromide. The probe was a PvuII-EcoRI restriction fragment of plasmid pBB-3NTS (Ward et al., 2000). Replication intermediates were quantified with Image Gauge and normalized against the 1N spot after subtraction of background signal for each area. Signals were corrected for incomplete release from α factor based on quantitative FACS analysis, and unspecific signal from quantification of the G1 samples was subtracted from the mid-late S and late S-G2 samples resulting in the plotted values.

Immunoblotting and Western Blot Quantification

Total cellular protein extracts were prepared by TCA precipitation from 1.5×10^7 cells, resolved on NuPAGE 4%–12% gradient gels (*Invitrogen*), and analyzed by standard western blotting techniques. Western blots were quantified with Image Gauge software (Science Lab). The percentage of Rad53 phosphorylation was calculated by dividing the level of (slower migrating) phospho-Rad53 by the level of total Rad53 (unmodified plus modified) for each time-point depicted in Figures 4D and 5C. The quantification shown in Figure S1D gives values of band intensities (normalized to a loading-control) in relation to their maximum values.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and one table and can be found with this article online at doi:10.1016/j.cell.2010.02.028.

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